Combined inhibition of nitrergic and prostanoid pathways in J774 macrophages


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Abstract

Objectives
Nitric oxide and prostaglandins are both implicated in the pathogenesis of inflammatory conditions such as rheumatoid arthritis (RA). The hypothesis that simultaneous inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX) was more effective than inhibition of either enzyme alone was tested.

Methods
J774 macrophages were pre-incubated with L-NAME and/or indomethacin, prior to activation with LPS (10 μg/ml).

Results
LPS significantly increased NO₂⁻, PGE₂ and TNF-α levels by 24h. Quantitative real-time PCR demonstrated a dose-dependent reduction in the expression of COX-2 in the presence of increasing doses of L-NAME. NO₂⁻ and PGE₂ production were inhibited in a dose-dependent manner by either indomethacin or L-NAME. Combined administration of L-NAME and indomethacin produced a significantly greater inhibition of NO₂⁻ and PGE₂ than either inhibitor alone.

Conclusion
The data supports the therapeutic potential of combined inhibition of the prostanoid and nitrergic systems as an anti-inflammatory treatment strategy and supports the progression of this work into models of arthritis.

Key words
Inflammation, nitric oxide (NO), prostaglandin E₂ (PGE₂), tumour necrosis factor-α (TNF-α).
Introduction

Nitric oxide (NO) and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) are two inflammatory mediators overproduced in arthritic joints (4). This is due to the high levels generated by the inducible enzymes in both pathways, namely inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Inflammatory stimuli, including endotoxins and numerous cytokines, induce the expression of both inducible isoforms at sites of inflammation or tissue injury. iNOS and COX-2 have previously been detected in synovial tissue, fluid and articular cartilage of arthritic joints (5-11). Despite the early promise of NOS inhibitors in suppressing the development of arthritis, there is uncertainty about their efficacy in treating established diseases (12-14). Similarly, drugs that inhibit PG synthesis have been found to be valuable in inhibiting inflammatory hyperaemia and reducing acute flares in rheumatoid arthritis (RA) (9, 11, 15) but have had little impact on disease progression (16).

The excessive production of NO elicits tissue damage partly through the production of the cytotoxic peroxynitrite (ONOO\textsuperscript{-}) (17-20). However, there are also constitutively expressed isoforms that generate low amounts of NO necessary for the regulation of numerous physiological processes, including blood pressure, platelet adhesiveness, gastro intestinal motility and neurotransmission (21).

Two isoforms of COX also exist for the formation of PGE\textsubscript{2}. The constitutive isoform, COX-1, typically plays a housekeeping role while the inducible COX-2 is known to play a central role in the inflammatory process. Both enzymes catalyse the conversion of arachidonic acid to prostanooids which are ultimately converted to the more stable PGs, prostacyclin and thromboxane A\textsubscript{2}. The anti-inflammatory action of non-steroidal anti-inflammatory drugs (NSAIDs) result from their inhibition of COX-2, whereas the adverse effects associated with this class of drugs can largely be attributed to inhibition of COX-1, a consequence of their lack of selectivity.

Both nitricergic and prostanooid systems are activated during inflammation, and various studies have investigated the interaction between these pathways and showed low levels of NO activate COX (5, 22) while higher levels appear to reverse the effect. Swierkosz (1995) (1) demonstrated J774 macrophages, stimulated with lipopolysaccharide (LPS), induces release of large amounts of NO which inhibited both the activity and induction of COX-2.

During chronic inflammation, cells of the immune system such as monocytes/macrophages infiltrate the synovial tissue. Previous studies have demonstrated that the abundance of macrophages in the inflamed synovium correlates with the severity of the RA. On activation by bacterial products or interferon-γ, these cells release a variety of catabolic cytokines, in particular tumour necrosis factor (TNF-α) (24). These inflammatory cytokines up regulate the enzymes which produce chemical mediators such as PG and NO (25, 26). The release of these substances results in vasodilatation and increased vascular permeability at the site of infection.

Given the known interaction between the nitricergic and prostanooid pathways, this study aims to establish if additional benefit is derived by the combined inhibition of both pathways simultaneously in macrophages, thereby offering an improved therapeutic strategy.

Methods and materials

Cell culture

The murine macrophage cell line, J774 (European Collection of Animal Cell Cultures (ECACC), Salisbury, Wiltshire, UK), were cultured using standard cell culture techniques. The cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM; Invitro-
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and, Paisley UK) and supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine and Penicillin/Streptomycin 50 µg/ml. Confluent cells were washed twice and resuspended in complete RPMI 1640 medium before seeding them onto 24-well plates.

An inflammatory response was induced by stimulating the cells with the bacterial toxin, lipopolysaccharide (LPS, 10 µg/ml). NOS and COX activity were inhibited by incubation of the cells with N\textsuperscript{G}-nitro-L-arginine (L-NAME) or indomethacin, respectively, 30 min prior to activation with LPS. Twenty-four hours later, the accumulation of TNF-α, nitrite (NO\textsubscript{2}⁻) and prostaglandins (PGE\textsubscript{2}) in the supernatants were measured as indices of the inflammatory response.

**Production of nitrite, TNF-α and PGE\textsubscript{2} in LPS-stimulated J774**

TNF-α is a key cytokine produced by macrophages on activation by LPS. The amount of TNF-α in the supernatant was calculated using an ELISA standard curve of mouse recombinant TNF-α, following the manufacturers protocol (Biosource International, Camarillo, CA, USA). Briefly, culture supernatants were added to 96-well plates pre-coated with a mouse TNF-α antibody. The absorbance of samples was measured at 450 nm, after detection with a streptavidin-horse radish peroxidase solution. NO production was measured as NO\textsubscript{2}⁻ levels using a spectrophotometric assay based on the Griess reaction. Briefly, culture supernatants were mixed with one volume of Griess reagent (0.1% naphthylethlenediamine and 1% sulfanilamide in 85% phosphoric acid). After 30 min, absorbance was read at 565 nm, and NO\textsubscript{2}⁻ concentration was calculated from a standard curve using sodium nitrite. PGE\textsubscript{2} was determined by ELISA (Cayman Chemical Company, Ann Arbor, MI, USA). The concentration of PGE\textsubscript{2} in cell supernatants was calculated from a standard curve of PGE\textsubscript{2}, measured at 410 nm (Dynex MRII).

**Quantitative RT-PCR**

Total RNAs of the J774 mouse macrophages were extracted using TRIzol reagent (5x10\textsuperscript{6} cells/ml, Sigma, UK) according to manufacturers procedure. First strand cDNA was made from total RNA using a reverse transcription kit (Promega, UK). Relative expression of COX-1 and 2 were determined by real-time quantitative RT-PCR using the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems, USA). The oligonucleotide sequences for primers and probes used are as follows: COX-1 forward primer: CAC CAG TCA TTC CCT GTT GTT ACT, reverse primer: CCA GGT CCA GAT CTC AGG GAT A, probe: TCC ATG CCA GAA CCA GGG TGT CTG (27); COX-2 forward primer: AGG TGT ATC CTC CCA CAG TCA AA, reverse primer: GGC ACC AGA CCA AAG ACT TCC T, probe: ACA CTC AGG TAG ACA TGA TCT ACC CTC CCC A (27); β-actin forward primer: TTC AA C A CC A CCA GCC ATG T, reverse primer: GTG GTA CGA CCA GAG GCA TAC A, probe: CGT AGC CAT CCA GCC TGT GTT GTC C (28). All Taqman probes were labelled at the 5’ end with 6-carboxy-flourescein (FAM) and at the 3’ end with a non- fluorescent dark quencher molecule. The comparative threshold cycle (C\textsubscript{T}) method was used for the relative quantification of gene expression as described in the user manual utilising β-actin RNA levels as an internal standard. Taqman reverse transcription reagents were used as described by manufacturer.

**Assessment of cell survival**

Cell viability was assessed by mitochondrial reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. After removal of the supernatant, the cells were incubated with MTT (5 mg/ml in PBS, pH 7.4 filter-sterilised) for 4 hours at 37°C. Medium was removed and the cells were solubilised in 10% sodium dodecyl sulphate (SDS), giving rise to a coloured product that can be detected at 540 nm using a spectrophotometer. The amount of coloured product is proportional to the number of living cells.

**Drugs**

All culture media and nutrients were from Invitrogen (Paisley, UK), and all other chemicals used were from Sigma (UK). L-NAME and indomethacin were dissolved in saline and sodium carbonate respectively.

**Statistical Analysis**

All results are expressed as percentage change ± S.D. compared to LPS-induced cells, and have been normalised against unstimulated cells. IC\textsubscript{50} data expressed as means and 95% confidence intervals. Comparison between group values was performed by one- or two-way repeated measures ANOVA, as indicated. Bonferroni multiple comparison tests were used post hoc for comparison of the different treatments, unless otherwise stated. n values refer to the number of tests performed for each group.

**Results**

**Effect of combined application of L-NAME and indomethacin on PGE\textsubscript{2} levels**

PGE\textsubscript{2} levels were increased significantly when LPS was applied to the cells (P<0.0001, 1-way ANOVA; n=4; Table I). PGE\textsubscript{2} production was progressively inhibited (Fig. 1A) by application of increasing concentration of L-NAME (IC\textsubscript{50} = 21.2 µM; 95% CI 18.7 to 23.6 µM) or indomethacin (IC\textsubscript{50} = 14.7 µM; 12.3 to 17.17 µM, 95% CI). Combined administration of L-NAME and indomethacin produced greater inhibi-

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**Table I. Basal and LPS-stimulated values of TNF-α, NO\textsubscript{2}⁻ and PGE\textsubscript{2}. (Values represent mean ± SD; statistical comparisons are by paired t-tests)**

<table>
<thead>
<tr>
<th>Inflammatory index</th>
<th>Control</th>
<th>LPS 10 mg/ml</th>
<th>Statistics</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>3.8 ± 0.4 ng/ml</td>
<td>981 ± 120 ng/ml</td>
<td>P &lt; 0.0001</td>
<td>8</td>
</tr>
<tr>
<td>NO\textsubscript{2}⁻</td>
<td>0.98 ± 0.04 µM</td>
<td>41.8 ± 7.9 µM</td>
<td>P &lt; 0.0001</td>
<td>13</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>42.3 ± 11.1 pg/ml</td>
<td>11040 ± 2100 pg/ml</td>
<td>P &lt; 0.0001</td>
<td>5</td>
</tr>
</tbody>
</table>
tion of PGE_2 production than either drug alone (Fig. 1B), 2-way ANOVA revealing a significant interaction (P = 0.009; n=4).

Effect of combined application of L-NAME and indomethacin on NO_2 levels
LPS stimulation of J774 cells resulted in a greater than 40-fold increase in NO_2 compared to unstimulated cells (P < 0.0001; Table I). The release of NO_2 was inhibited in a concentration-dependent manner (Fig. 2A) by prior incubation with either L-NAME (IC_{50} = 1.55 mM; 1.2 to 1.9 mM, 95% CI) or indomethacin (IC_{50} = 0.19 mM; 0.12 to 0.26 mM, 95% CI). Various combinations of these inhibitors were then added to the stimulated cells (Fig. 2B) to assess the effects of inhibiting the nitricergic and prostanoid pathways in combination. When L-NAME and indomethacin were applied in combination, they reduced NO_2 levels significantly compared to LPS and also to independent administration of either drug alone, significant interaction being revealed by 2-way ANOVA (P = 0.0007; n=4).

Effect of sub-maximal concentrations of L-NAME and indomethacin on TNF-α levels
As expected, TNF-α levels were significantly increased in LPS-stimulated cells (P < 0.0001; paired t-test; n=8; Table I), with only a slight, non-significant reduction when sub-maximal concentrations of L-NAME (10^{-3} M) and indomethacin (10^{-4} M) were applied individually (99.7 ± 12.4% and 92.9 ± 11.7% respectively). In the combination inhibitor group, the LPS-induced rise in TNF-α (73.2 ± 10.1 %) was significantly reduced compared to untreated LPS-stimulated cells (P < 0.001), or either the L-NAME (P < 0.001) or indomethacin (P < 0.05) group (comparison made by Bonferroni post-hoc tests).

Changes in the expression of COX mRNA expression in response to LPS and L-NAME
Analysis of transcripts showed that the housekeeping gene β-actin mRNA C_T values were constant and not statistically different between treatment groups (C_T = 26.6 ± 0.9; n=10). Thus, β-actin expression did not significantly alter between treatments supporting the statement that drug treatments did not elicit a cytotoxic response. Quantification of COX-2 in non-induced J774 cells indicated low-level expression (Fig. 3), while COX-1 mRNA was not detected after 40 cycles of amplification. Both COX-1 and COX-2 mRNA were detected by RT-PCR (data not shown). Upon stimulation with LPS there was a greater than 80-fold increase in COX-2 expression (P < 0.05). Pretreatment with L-NAME and LPS caused a dose-dependent reduction in COX-2 mRNA expression, whilst L-NAME in unstimulated macrophages did not affect COX-2 expression.

Cell viability
Treatment of J774 cells with indomethacin and/or L-NAME did not result in any significant loss of cell viability when quantified using the MTT assay.

Discussion
The cell culture system provides a suitable model of inflammation in which to test the possible benefits of combined inhibition, since it avoids the systemic effects of using non-selective NOS and COX inhibitors in vivo. The results presented support the hypothesis that combined inhibition of the NOS/COX pathways in vitro has potential benefit as an anti-inflammatory strategy, while clearly demonstrating the sensitivity of the prostanoïd system over the nitricergic system.

As previous studies have shown, LPS was found to significantly increase levels of NO_2, PGE_2 and the cytokine TNF-α in the J774 murine macrophage cell line (1, 29-32). The increase in NO_2 and PGE_2 levels were found to parallel the induction of iNOS and COX-2, as determined by RT-PCR and real-time PCR for the expression of
COX-2 mRNA. The increased NO₂ levels were attenuated in a concentration dependent manner by the non-selective NOS inhibitor, L-NAME. Interestingly, the COX inhibitor, indomethacin, also inhibited NO production from these cells by a comparable magnitude (Fig. 1A). Similarly, L-NAME was as effective as indomethacin in reducing PGE₂ levels (Fig. 3A). These findings underline the strength of the cross talk between nitricergic and prostanoid pathways in macrophages as reported by others (1, 22, 33, 34). Interaction between these pathways is further supported by our finding that when the two drugs were applied in different combinations, NO₂ and PGE₂ were reduced significantly below the values obtained by individual drug application (Fig. 1B, 2B). Interestingly, real-time PCR clearly showed COX-2 mRNA expression was reduced in the presence of LPS and L-NAME, supporting the effect of L-NAME on PG production. Different concentrations of the inhibitors were required for blocking the nitricergic and prostanoid pathways, which may reflect the greater sensitivity of the prostanoid system.

TNF-α is a macrophage-derived cytokine which was used as a third indicator of the inflammatory response. TNF-α is a potent signal transmitter peptide derived primarily from cells of the immune system and is responsible for inducing a range of activities including activation iNOS and COX-2. In the presence of LPS, J774 released significantly large amounts of this cytokine. Administration of either L-NAME or indomethacin alone did not reduce TNF-α levels, but when combined at submaximal concentrations, the cytokine levels were significantly attenuated. This is an interesting result as TNF-α is thought to play a pivotal role in inflammatory conditions. Comparing the results of combined inhibition of NO₂ with that of TNF-α, demonstrates internal consistency and supports the results of the MTT assay (data not shown), which demonstrates that L-NAME and indomethacin-induced NO inhibition were not due to cell toxicity as the same concentrations were used and did not elicit a response in TNF-α levels.

Several studies to date have demonstrated that NO interacts with the COX pathway, however the nature of this interaction is the subject of ongoing debate (1, 35). NO has been demonstrated to both inhibit (36,37) and stimulate (22, 38) COX activity, and the nature of the interaction has been suggested to be concentration-dependent. The mechanisms proposed for NO activation of COX include the production of superoxide anions and consequently the cytotoxic peroxynitrite (39, 40) or by direct binding to the haem moiety of COX (1). Activated macrophages, in addition to increasing the synthesis of NO, also generate reactive oxygen species, such as superoxide anions, that react spontaneously with NO to form the cytotoxic peroxynitrite. This has been suggested to act as an activator of COX activity (41). Alternatively, NO has been shown to bind to the haem moiety of COX at high concentrations, and reduce it to the ferrous-inactive form of COX (1) or to nitrosylate cysteine groups within COX (42).

With respect to PGE₂ actions on NO, the literature remains divided and complex. Previous studies have demonstrated PGE₂ suppresses TNF-α, which would have an indirect effect of suppressing iNOS activation (31, 43), while other studies have shown PGE₂ directly regulates NO production (2, 29, 44). Swierkosz (1995) (1) observed that COX metabolites had no effect on NOS activity whilst NO was found to inhibit both COX activity and induction. Furthermore, agents that increase the second messenger cyclic AMP (cAMP) e.g. PGE₂, have been shown to inhibit cytokine production by macrophages (30, 32). The molecular mechanisms by which prostanoids modulate iNOS expression are not yet understood.

Fig. 2. Dose-dependent effect of L-NAME or indomethacin on NO₂ levels. (a) Accumulation of NO₂ in the medium bathing J774 cells stimulated with LPS (10 mg/ml) for 24 h was inhibited by L-NAME ( ) and indomethacin ( ) (P < 0.0001, n = 4, 1-way ANOVA for both); (b) 3D plot showing combined L-NAME and indomethacin inhibition of LPS-induced NO₂ production. (means ± SD; n = 4). 2-way ANOVA revealed a significant interaction of combination therapy (P = 0.0007).
clear. NO has been demonstrated to regulate activity of COX enzymes, enhancing or inhibiting their synthesis. An important pathway linking the NOS and COX systems is their transduction by Nuclear Factor-κB (NF-κB). NF-κB plays a critical role in the transcriptional regulation of inflammation. NF-κB is increased in activated cells by proteasomes, allowing NF-κB nuclear translocation and gene activation. Previous studies have shown inhibition of NF-κB prevents iNOS and COX-2 protein expression.

Activation of NF-κB is increased in several chronic inflammatory diseases and is responsible for the enhanced expression of many proinflammatory gene products. The link among TNF-α, nitric oxide, prostaglandins and NF-κB could have considerable importance for regulation of inflammation.

In conclusion, the clear plane of interaction (Fig. 1B & 2B) when inhibiting prostanooid and nitrergic systems simultaneously supports the anti-inflammatory potential of combination therapies targeting both these systems, and directs the progression of this work into animal models of arthritis.

**Acknowledgements**

We wish to thank Dr. Sharron Dolan for her technical assistance.

**References**


